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PTHrP 1-141 and 1-86 Increase In Vitro Bone Formation

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Abstract

Background—Parathyroid hormone-related protein (PTHrP) has anabolic effects in bone, which has led to the clinical use of N-terminal fragments of PTHrP and PTH. Since 10-20% of fractures demonstrate healing complications and osteoporosis continues to be a debilitating disease, the development of bone-forming agents is of utmost importance. Due to evidence that regions of PTHrP other than the N-terminus may have bone-forming effects, this study was designed to compare the effects of full-length PTHrP 1-141 to N-terminal PTHrP 1-86 on in vitro bone formation.

Materials and methods—MC3T3-E1 pre-osteoblasts were treated once every 6 days for 36 days with 5, 25, and 50 pM of PTHrP 1-141 or 1-86 for 1 or 24 hours. Cells were also treated after blocking the N-terminus, the nuclear localization sequence (NLS), and the C-terminus of PTHrP, individually and in combination. Area of mineralization, alkaline phosphatase (ALP), and osteocalcin (OCN) were measured.

Results—PTHrP 1-141 and 1-86 increased mineralization after 24-hr treatments, but not 1-hr. PTHrP 1-141 was more potent than 1-86. Treatment with PTHrP 1-141 for 24-hr, but not 1-86, resulted in a concentration-dependent increase in ALP, with no effect after 1-hr. Exposure to both peptides for 1- or 24-hrs induced a concentration-dependent increase in OCN, with 24-hr exceeding 1-hr. Antibody blocking revealed that the NLS and C-terminus are anabolic.

Conclusions—Both PTHrP 1-141 and 1-86 increased in vitro bone formation; however, PTHrP 1-141 was more effective. The NLS and C-terminus have anabolic effects distinct from the N-terminus. This demonstrates the advantage of PTHrP 1-141 as a skeletal anabolic agent.

Keywords

PTHrP 1-141; PTHrP 1-86; C-terminus; NLS; MC3T3-E1; mineralization; LCC15-MB

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INTRODUCTION

Parathyroid hormone-related protein (PTHrP) was first discovered as the principal endocrine factor in the development of humoral hypercalcemia of malignancy; however, it serves a vital role in many normal physiologic processes, including fetal bone development, cellular growth and differentiation, and mesenchymal-epithelial interactions [1-3]. The primary effects of PTHrP are attributed to its N-terminus, where it is equipotent with N-terminal PTH at the common PTH/PTHrP receptor (PTH1R) [4]. However, in contrast to PTH, PTHrP is not normally present in circulation, indicating that PTHrP acts primarily in an autocrine, paracrine, and intracrine role, rather than in an endocrine manner [5]. PTHrP is a prohormone capable of undergoing post-translational modification into several discrete peptides, each believed to act through its own specific receptor to elicit a spectrum of distinct, independent physiological responses. In addition to the N-terminus, there is evidence that PTHrP has three other functional domains: 1) a mid-region, important for transplacental calcium transport, 2) a nuclear localization sequence (NLS), involved in nuclear transport with inhibition of apoptosis and promotion of cellular proliferation and differentiation, and 3) a C-terminus, historically associated with inhibiting osteoclastic bone resorption [6-9]. While the specific peptides and receptors of PTH are well-defined, less is known regarding the secretory fragments of PTHrP or their respective receptors.

Since the discovery of the anabolic effects of ancestrally-related PTH in bone, PTHrP has also received attention as a potential skeletal therapeutic agent [10]. The bone-forming effects of both peptides depend on the dose and pattern of administration, with the greatest increase in skeletal accretion generated by brief daily exposure, where at least initially, de novo bone formation is preferentially increased by actions on cells of the osteoblast lineage [10]. Due to the current limitations of cost and patient compliance associated with daily administration of PTH, alternative treatment regimens are being investigated, of which once-weekly administration has also proven effective [11]. In addition, recent studies have indicated that the other functional domains of PTHrP may have anabolic effects in bone distinct from, or that contribute to, those elicited by the N-terminus [12,13]. This is of particular interest since it has been suggested that there is no difference in the biological effects of full-length PTH and PTHrP in osteoblasts compared to their equipotent N-terminal fragments [4,14]. However, the use of full-length PTHrP 1-141 as a skeletal anabolic agent may be advantageous since; 1) cells of the osteoblast lineage not only express PTH1R but may secrete PTHrP, establishing the importance of PTHrP in the commitment of osteoprogenitor cells, skeletal patterning, and the maintenance of skeletal integrity, 2) PTHrP may bind alternative receptors for regions of the peptide other than the N-terminus in addition to being the natural ligand for PTH1R, and 3) PTHrP may be both more stable and anabolic than PTH 1-34 or 1-84 and PTHrP 1-36 or 1-86 [15-18]. Despite these potential advantages, few studies have investigated the anabolic effects of full-length PTHrP 1-141 due to its lack of commercial availability and expense.

Since approximately 10-20% of fractures demonstrate healing complications and osteoporosis continues to be a debilitating disease, the development of locally-delivered or systemic bone-forming agents is of utmost importance [19]. Therefore, due to the potential advantages of full-length PTHrP 1-141 as a skeletal anabolic agent, the purpose of this study was to compare the effects of full-length PTHrP 1-141 and N-terminal PTHrP 1-86 on in vitro bone formation. Here we show that both PTHrP 1-141 and 1-86 increased in vitro bone formation. However, PTHrP 1-141 was more potent due to the presence of the NLS and C-terminus, which possess complimentary anabolic effects to the N-terminal domain of PTHrP.

MATERIALS AND METHODS

Transfection of the LCC15-MB cell line

The HARA epithelial cell line, which is derived from a human lung squamous cell carcinoma, served as a source of PTHrP 1-141 mRNA (a generous gift from Dr. H. Iguchi, National Kyushu Cancer Center, Fukuoka, Japan) [20]. Total RNA was extracted and full-length (−36 ~ +141) cDNA amplified by RT-PCR. Primers were 5′-CTA TAG GCT AGC GAG ACG ATG CAG CGG AGA-3′ (forward) and 5′-CTA TAG CTC GAG TCA ATG CCT CCG TGA ATC-3′ (reverse), with underlined sequences representing NheI and XhoI restriction sites. Sequences were cloned into a pcDNA3.1 (+) expression vector (Invitrogen Corp., Carlsbad, CA). Both the recombinant plasmid and the empty vector were stably transfected into human LCC15-MB cells [21], which secrete negligible PTHrP 1-141, to generate the LCC15-PTHrP 1-141 and LCC15-vector cell lines, respectively. Stable transfectants were selected with 1 mg/ml G418 sulfate for 3 weeks and maintained in Dulbecco's modified Eagle's media (DMEM)/F-12, 10% fetal bovine serum (FBS), and 400 μg/ml G418 sulfate at 37 °C and 5% CO_2 (Invitrogen). The presence of both expression vectors was confirmed by real-time PCR, sequencing, and western blot analysis.

Conditioned media preparation and PTHrP 1-141 quantification

The LCC15-PTHrP 1-141 and LCC15-vector cell lines were plated in T-75 flasks (BD Falcon, BD Biosciences, Bedford, MA) and maintained in DMEM/F-12, 10% FBS, 250 U/ml penicillin, 250 μg/ml streptomycin, and 2 mM L-glutamine (standard growth media) at 37 °C and 5% CO_2. At confluence, media was aspirated, cells washed with phosphate-buffered saline (PBS, Invitrogen), and serum-free media applied. After 48 hrs, conditioned media (CM) was aspirated, centrifuged at 290 *g* for 6 minutes at 4 °C to remove cellular debris, supernatant divided into aliquots, and LCC15-PTHrP 1-141 CM (now referred to as PTHrP 1-141) and LCC15-vector CM (now referred to as vector) frozen at −80 °C. The concentrations of PTHrP 1-141 in 5 batches of each CM were measured with a two-site immunoradiometric assay (IRMA) (DSL-8100, DSL, Inc., Webster, TX). The IRMA uses antibodies specific for the N-terminus (amino acids 1-40) and mid-region (amino acids 57-80) of PTHrP. The lowest PTHrP concentration detectable by the assay was 0.30 pM.

Cell viability assays and conditioned media protein quantification

To determine if transfection with PTHrP 1-141 resulted in differences between CM other than PTHrP 1-141, we compared the viability of LCC15-PTHrP 1-141 and LCC15-vector cells and protein concentrations in their respective CM. Both cell lines were plated in triplicate at an initial density of 2,500 cells per well in a total volume of 100 μl DMEM/F-12, 250 U/ml penicillin, 250 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen) in a 96-well microplate and maintained at 37 °C and 5% CO_2. After 24, 48, and 72 hrs, 15 μl of MTT dye solution was applied and incubated for 4 hrs (CellTiter 96® Non-Radioactive Cell Proliferation Assay, Promega Corp., Madison, WI). The reaction was terminated by incubation with 100 μl of stop solution for 1 hr and the absorbance of formazan measured at 570 nm in a plate reader (SOFTmax® PRO v3.1, Molecular Devices Corp., Sunnyvale, CA). Protein concentrations in 5 batches of PTHrP 1-141 and vector were measured using a colorimetric assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Inc., Hercules, CA). In a 96-well microplate, 200 μl of dye reagent and 1 μl of CM were combined and the absorbance measured at 595 nm.

Western blot analysis

LCC15-PTHrP 1-141 and LCC15-vector cell lines were plated in a 6-well plate (Costar®, Corning Inc., Corning, NY) and grown until confluent. Prior to harvest, media was removed

and cells were treated with either DMEM/F-12 standard growth media or standard growth media containing 1 μl/ml of a Golgi blocker to block PTHrP secretion (Golgi Plug™, BD Biosciences, Franklin Lakes, NJ). Cells were treated for 5 hrs at 37 °C, washed once with PBS and lysed in SDS lysis buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 nM dithiothreitol (DTT), 0.01% w/v bromophenol blue) (Invitrogen). Whole cell lysates were boiled at 100 °C in water for 5 minutes and 75 μg of protein was separated on an 18% glycine-based SDS-PAGE gel at 110 volts for 1.5 hrs (Invitrogen). Samples were transferred to nitrocellulose membranes at 90 volts for 1 hr, blocked in 5% milk/PBS-0.1%Tween-20 (PBS-T) (Invitrogen) for 30 minutes at room temperature, and incubated with mouse primary monoclonal anti-human antibody to PTHrP 38-64 (Calbiochem, La Jolla, CA). Primary antibody was diluted 1:1000 in 5% milk/PBS-T and incubated overnight at 4 °C. The membrane was washed 3 times for 5 minutes using PBS-T. Secondary antibody, sheep anti-mouse horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), was also diluted 1:1000 in 5% milk/PBS-T.

Evaluation of PTHrP 1-141 stability

To assess the in vitro degradation of full-length PTHrP 1-141, we exposed MC3T3-E1 pre-osteoblasts, which secrete negligible PTHrP 1-141, and LCC15-vector cells to the peptide and measured changes in PTHrP 1-141 concentrations for 7 days. Pre-osteoblastic MC3T3-E1 cells and LCC15-vector cells were maintained in alpha-modified minimum essential media (α-MEM) standard growth media, which contains no detectable PTHrP 1-141, at 37 °C and 5% CO_2. Cells were plated in triplicate at an initial density of 50,000 cells per well in 3 ml of standard growth media in a 6-well plate (Corning). At confluence, wells were treated with 300 pM of PTHrP 1-141 in standard growth media. Aliquots (210 μl) of media were collected at baseline (0 hrs), 6, 12, and 24 hrs and 7 days after treatment, and an equivalent volume of standard growth media, which has undetectable PTHrP, replaced into the wells. The concentration of PTHrP 1-141 was measured at each time point with a two-site IRMA, accounting for the dilution factor at each time point.

MC3T3-E1 pre-osteoblast mineralization assays

Mineralization was the primary outcome measure due to the fact that MC3T3-E1 pre-osteoblasts undergo a temporal developmental pattern modelling in vivo bone formation. Power analysis revealed that three individual wells for each treatment group and treatment duration would achieve a power of 0.8 at $\alpha = 0.05$. Cells were plated at an initial density of 50,000 cells per well in 6-well plates (Corning) and 3 individual wells were grouped and treated once every 6 days with either: 1) osteogenic media (OM); 2) 5, 25, and 50 pM of PTHrP 1-141 in OM for treatment durations of 1- and 24-hr; 3) 5, 25, and 50 pM of human recombinant PTHrP 1-86 (Abcam Inc., Cambridge, MA) in vector (equivalent to the respective volumes of PTHrP 1-141) and OM for treatment durations of 1- and 24-hr; and 4) equivalent volumes of vector (referred to as equivalent pM) corresponding to the volumes required to obtain PTHrP 1-141 concentrations of 5, 25, and 50 pM in OM for treatment durations of 1- and 24-hr (Fig. 1). The human recombinant PTHrP 1-86 was produced in an *E. coli* expression system. Osteogenic media, consisting of α-MEM with Glutamax™ (Invitrogen), 10% FBS, 250 U/ml penicillin, 250 μg/ml streptomycin, 250 μM ascorbic acid (Fisher Scientific International, Inc., Fair Lawn, NJ), and 10 mM β-glycerophosphate (MP Biomedicals, Inc., Solon, OH), was applied after each treatment duration and changed in all groups every third day of each 6-day cycle (Fig. 1). At ~90% confluence, triplicate wells were treated with 2.5 ml of respective treatment media (day 0). Conditioned media represented 0.40, 2.0, and 4.0% of the total volume of treatment media for 5, 25, and 50 pM, respectively. Media was aspirated and frozen at −80 °C during each 6-day cycle for 36 days.

To determine the mechanism by which differences in bone formation indices following treatment with either PTHrP 1-141 or 1-86 occurred, we performed additional mineralization assays assessing the effects of blocking specific regions of both peptides. Ten treatment groups were established (8 for PTHrP 1-141 and 2 for PTHrP 1-86) and mineralization assays exposed to 50 pM of treatment media for 24-hr once every 6 days. Conditioned media represented 8.0% of the total volume of treatment media. Before treating the mineralization assays with PTHrP 1-141, the PTHrP 1-141 treatment media was incubated for 1-hr with each of the following, both individually or all possible combinations thereof: 1) an anti-N-terminal blocking antibody (anti-PTHrP 1-86, Santa Cruz Biotechnology), 2) an anti-NLS blocking antibody (anti-PTHrP 93-107, AbD Serotec, Raleigh, NC), 3) an anti-C-terminal blocking antiserum (anti-PTHrP 107-121, ALPCO Diagnostics, Salem, NH), or 4) nothing (control). Treatment media containing PTHrP 1-86 was incubated for 1-hr with either 1) an anti-N-terminal antibody or 2) nothing (control). Antibodies and antiserum were applied at a 5-fold excess to the concentration of PTHrP.

von Kossa staining and mineralization quantification

After 36 days, wells were fixed with 95% ethanol for 15 minutes, rinsed, and dried. Wells were treated with 5% silver nitrate (Sigma-Aldrich, Inc., St. Louis, MO) in the dark for 20 minutes, rinsed, and treated with 0.5% hydroquinone (Acros Organics, Geel, Belgium) under ultraviolet light for 5 minutes. Wells were then rinsed, treated with 5% sodium thiosulfate (Fisher), rinsed, and dried. Plates were imaged with a 12.5 megapixel resolution 16-bit digital camera (Model D2X, Nikon Instruments Inc., Melville, NY) and imported into image analysis software (Image-Pro Plus v5.0, Media Cybernetics, Inc., Bethesda, MD). Area of mineralization (mm^2) was calculated from the automated tracings of the von Kossa stained regions within the central 85% of each well, visually-verified for accuracy in a blinded manner.

Alkaline phosphatase (ALP) assay

ALP activity was measured in the media of each well at days 6, 12, 18, 24, 30, and 36 and in vector and PTHrP 1-141 using a *p*-nitrophenyl phosphate (*p*NPP) colorimetric assay (SensoLyte™ pNPP Alkaline Phosphatase Assay Kit, Anaspec, Inc., San Jose, CA). The assay had a detection limit of 0.01 ng. In a 96-well microplate, 50 μl of *p*NPP reaction mixture and 50 μl of media were incubated for 30 minutes, reaction terminated with stop solution, and absorbance measured at 405 nm. ALP activities were normalized to total secreted protein using the previously described colorimetric assay (Bio-Rad).

Osteocalcin (OCN) assay

OCN concentrations were measured in the media of each well at day 36 and in vector and PTHrP 1-141 using a mouse-specific sandwich ELISA (Mouse Osteocalcin EIA kit, Biomedical Technologies Inc., Stoughton, MA). We selected day 36 to measure OCN secretion due to the fact it is a late marker of osteoblast differentiation and while undetectable in pre-osteoblasts, it is abundantly expressed in mature, mineralizing osteoblasts [22]. The assay had a sensitivity of 1 ng/ml. In a 96-well microplate, 100 μl of OCN antiserum and 25 μl of media were incubated at 4 °C for 24 hrs. Wells were rinsed 5 times with PBS wash buffer and exposed to 100 μl streptavidin-horseradish peroxidase for 30 minutes. Wells were rinsed, 100 μl of a 50:50 mix of tetramethyl benzidine:hydrogen peroxide applied, and incubated in the dark for 15 minutes. The reaction was terminated with 100 μl of stop solution and absorbance measured at 450 nm. Osteocalcin concentrations were normalized to total secreted protein using the previously described colorimetric assay (Bio-Rad).

Statistical analysis

All values are represented as mean ± 1 standard deviation. All data was normally distributed or of equal variance, except CM PTHrP 1-141 concentrations, which were compared with a Mann-Whitney test. Cell viability was compared between the LCC15-PTHrP 1-141 and LCC15-vector cell lines over 24, 48, and 72 hrs with a 2-way repeated measures ANOVA and a Bonferroni post-hoc analysis. Protein concentrations in vector and PTHrP 1-141 were compared with a Student's t-test. To assess the in vitro stability of PTHrP 1-141, concentrations were compared between time points with a 1-way RM-ANOVA and a Dunnett's post-hoc analysis within the MC3T3-E1 and LCC15-vector groups. Osteogenic indices were compared 1) between the OM, vector, PTHrP 1-86, and PTHrP 1-141 treatment groups, within each concentration, and 2) between concentrations within the vector, PTHrP 1-86, and PTHrP 1-141 treatment groups, within each treatment duration, with a 1-way ANOVA and a Newman-Keuls post-hoc analysis. Osteogenic indices were also compared within each concentration of the vector, PTHrP 1-86, and PTHrP 1-141 treatment groups between treatment durations with a 2-way ANOVA and a Bonferroni post-hoc analysis. Osteogenic indices were compared between PTHrP 1-141 and 1-86 in the presence or absence of all individual antibodies and all potential blocking combinations using a 1-way ANOVA and a Newman-Keuls post-hoc analysis. Reproducibility of the results were verified by performing three sets of independent mineralization assays assessing the effects of exposure concentration and duration and two sets of independent mineralization assays assessing the effects of region-specific blockage, with the cohorts treated in parallel. All analyses were performed with commercial software (GraphPad Prism v5.00, GraphPad Software, Inc., San Diego, CA) and the statistical significance established at $P < 0.05$.

RESULTS

Stable transfection of LCC15-MB cells resulted in over-expression of PTHrP 1-141

Stable transfection of LCC15-MB cells resulted in increased secretion of PTHrP 1-141, with 1,200 ± 310 pM present in PTHrP 1-141 and 3.3 ± 0.9 pM present in vector ($P = 0.0079$). Western blot analysis of whole-cell extracts detected the presence of a single isoform of PTHrP, 1-141, only in the LCC15-PTHrP 1-141 cell line (Fig. 2). For this study, a single batch of vector and PTHrP 1-141 medium was selected for assessing the effects of exposure concentration and duration and another batch for assessing the effects of region-specific blocking antibodies. The concentration of PTHrP 1-141 was 1,260 in PTHrP 1-141 and 2.0 pM in vector in the batches used in our assessment of the effects of exposure concentration and duration and 670 pM in PTHrP 1-141 and 3.1 pM in vector in the batches used in our assessment of the effects of region-specific blockade. Therefore, equivalent volumes of vector to 5, 25, and 50 pM of PTHrP 1-141 treatment media contained 0.008, 0.04, and 0.08 pM of PTHrP 1-141 in the former and equivalent volumes of vector to 50 pM of PTHrP 1-141 treatment media contained 0.2 pM of PTHrP 1-141 in the latter. Protein concentration did not differ between CM and there was no detectable ALP activity or OCN secretion in both CM. Viable cell number and proliferation did not differ between the LCC15-PTHrP 1-141 and LCC15-vector cells in culture from 24 to 72 hours.

PTHrP 1-141 was stable for 24 hours when exposed to MC3T3-E1 pre-osteoblasts and for 7 days when exposed to LCC15-vector cells

There was no statistical difference in the concentration of PTHrP 1-141 at baseline (0 hrs), 6, 12, and 24 hrs after treatment when exposed to MC3T3-E1 pre-osteoblasts; however, the concentration significantly decreased by 76% at day 7 ($P < 0.01$). In contrast, the concentration of PTHrP 1-141 was maintained for 7 days when exposed to LCC15-vector cells.

PTHrP 1-141 and 1-86 increased mineralization

PTHrP 1-141 (5, 25, and 50 pM) and 1-86 (5 pM) significantly increased the mineralization of MC3T3-E1 pre-osteoblasts after 24-hr of treatment once every 6 days compared to 1-hr of treatment ($P < 0.05$). PTHrP 1-141 induced greater mineralization compared to PTHrP 1-86 at 50 pM only ($P < 0.05$, Fig. 3A). There was a mild, but significant induction of mineralization by vector alone after 24-hr of treatment ($P < 0.05$, Fig. 3A). There was no significant effect on mineralization elicited by either analog after treatment for 1-hr other than a mild decrease after treatment with 5 pM of PTHrP 1-141 compared to vector ($P < 0.05$, Fig. 3B).

Region-specific blockade of PTHrP 1-141 had no effect on mineralization; however, there was a significant decrease when either the NLS and C-terminus combined or the complete peptide was blocked ($P < 0.05$, Fig 6A). All PTHrP 1-141 blocking combinations resulted in significantly greater mineralization than PTHrP 1-86 except for when there was blockade of the NLS and C-terminus combined or the complete peptide ($P < 0.001$, Fig. 6A).

PTHrP 1-141 increased ALP activity

At day 18, the peak of ALP activity in MC3T3-E1 pre-osteoblasts in culture, 24-hr of treatment with PTHrP 1-141 resulted in a concentration-dependent increase in ALP activity, exceeding PTHrP 1-86 ($P < 0.05$, Fig. 4A). Similar to mineralization, greater ALP activity occurred after 24-hr of treatment with PTHrP 1-141 ($P < 0.05$), with no effect elicited by 1-hr of treatment with the peptide (Fig. 4B). Treatment with PTHrP 1-86 for 24-hr had no effect on ALP activity when compared to 1-hr except at 5 pM ($P < 0.05$); however, there was no effect of PTHrP 1-86 when compared to controls.

All blocking combinations significantly decreased ALP activity when compared to PTHrP 1-141 except for when the N-terminus alone was blocked ($P < 0.01$, Fig 6B).

PTHrP 1-141 and 1-86 increased OCN secretion

PTHrP 1-141 and 1-86 induced a concentration-dependent increase in OCN secretion after 24-hr of treatment, and the effects were greater than 1-hr of treatment ($P < 0.001$, Fig. 5A**)**. There were mild increases in OCN secretion after 1-hr of treatment with both analogs ($P < 0.05$, Fig. 5B).

Blockade of PTHrP 1-141 resulted in no difference in OCN secretion when compared to PTHrP 1-141; however, there was a significant decrease after combined blocking of the N- and C-terminus, the NLS and C-terminus, and when PTHrP 1-86 underwent N-terminal blockade ($P < 0.05$, Fig 6C). Complete blocking of PTHrP 1-141 resulted in significantly less OCN secretion than all other combinations ($P < 0.05$) and both PTHrP 1-141 and 1-86 ($P < 0.001$, Fig 6C).

DISCUSSION

The goals of this study were to compare the effect of various concentrations and treatment durations of PTHrP 1-141 and 1-86 once every 6 days on in vitro bone formation and elucidate which regions of PTHrP were responsible for differences between the peptides. Both peptides were demonstrated to be anabolic. In addition, the NLS and C-terminus are capable of eliciting anabolic effects distinct from, or complimentary to, the N-terminus of the peptide.

Previous studies have demonstrated that that there is no difference in the biological effects of full-length PTH and PTHrP in osteoblasts compared to their equipotent N-terminal

fragments [4,14]. These previous findings are of importance in our study for two reasons. First, we used PTHrP 1-86 to induce anabolic effects in bone similar to N-terminal PTH 1-34 [14]. Second, full-length PTHrP 1-141 has important differences compared to PTHrP 1-86. PTHrP 1-141 is of particular significance since it contains both the NLS (amino acids 87-106) and C-terminus (amino acids 107-141) [7-9]. The physiologic relevance of the NLS and C-terminus were initially determined in *Pthrp* and *Pth1r* knockout mice, which differed from each other in their phenotype [23,24]. This discovery demonstrated that these domains of PTHrP have effects distinct from those mediated by the N-terminus. Specifically in bone, full-length PTHrP 1-141 inhibits osteoclastic bone resorption due to the actions of the C-terminus [9]. However, further in vitro studies have revealed that C-terminal PTHrP 107-139 promotes osteoblast proliferation and survival and intermittent treatment stimulates osteoblast differentiation similar to N-terminal PTHrP 1-36 [12,13]. In our laboratory, we have generated a transgenic mouse lacking the mid-region, NLS and C-terminus of PTHrP and another laboratory has independently developed a transgenic mouse lacking the NLS and C-terminus [25]. Our mice have decreased skeletal mineralization, reduced lineage commitment of osteoprogenitor cells, and decreased osteoblast number. These findings support that the anabolic effects of PTHrP may not be restricted solely to its N-terminal domain, with the additional domains of PTHrP potentially have additive or synergistic effects in bone formation and skeletal development. However, despite growing evidence of the positive effects of functional regions of PTHrP other than the N-terminus on bone mass, alternative receptor systems for these domains have yet to be fully characterized.

In our study, PTHrP 1-141 increased in vitro bone formation in a manner equivalent to or exceeding PTHrP 1-86. To elucidate which domains of PTHrP, or combinations thereof, were responsible for the differences, we evaluated the effects of region-specific blocking antibodies on bone formation indices. We have shown that the NLS and C-terminus of PTHrP have complementary or pro-osteogenic effects distinct from the N-terminus. In addition to when the NLS and C-terminus are present in full-length PTHrP 1-141, when each are combined with the N-terminus, there is a significant increase in mineralization when compared to PTHrP 1-86 alone. It has been reported that PTH1R expression increases during the second half of the culture period (Days 18 - 36) in MC3T3-E1 mineralization assays, just at or after peak ALP activity [26]. Our findings support that the NLS and C-terminus of PTHrP, likely acting through alternate receptors, are responsible for PTHrP 1-141 increasing ALP activity when compared to PTHrP 1-86. In addition, during the upregulation of PTH1R expression after day 18, these regions display complementary effects to the N-terminus, ultimately resulting in increased bone formation when compared to PTHrP 1-86.

We selected 1-hr as our shortest treatment duration, to mimic in vivo pulsatile dosing, and 24-hr as our longest, which coincides with the stability of full-length PTHrP 1-141 in vitro [27]. In a previous report, osteoblast differentiation was inhibited by treatment with PTH 1-34 (12 nM) for 1-hr once every 48 hrs, stimulated by treatment for 6-hr once every 48 hrs, and inhibited by continuous treatment for 48 hrs [28]. This suggests there were differential effects that depended upon treatment duration. Similar to these findings, there was a trend towards decreased mineralization and ALP activity after 1-hr of treatment with both PTHrP 1-141 and 1-86 in our study. In contrast, we found that 1-hr of treatment with PTHrP 1-141 and 1-86 resulted in a concentration-dependent increase in OCN secretion. This is of interest because while OCN is a late osteogenic biomarker, it is also one of the most specific [22]. Therefore, this suggests that 1-hr of treatment once every 6 days may have subtle effects on osteoblast differentiation despite the fact there was no increase in mineralization or ALP activity. However, our study used lower concentrations (5 to 50 pM) of PTHrP 1-141 and 1-86 and a longer cycle length (6 days versus 48 hrs), which resulted in less frequent treatments.

The development of cell lines that overexpress specific PTHrP isoforms without affecting cell viability has been previously reported, and these cells secrete PTHrP in a constitutive manner [29,30]. We also produced full-length PTHrP 1-141 without affecting cell viability or CM protein concentrations. Therefore, we speculate that differences other than the presence of absence of full-length PTHrP 1-141 between CM are small. However, to control for any potential differences that might exist, we placed PTHrP 1-86 in vector for the PTHrP 1-86 treatment group and used vector alone as a control in addition to OM. To measure the stability of full-length PTHrP 1-141 in vitro, we added PTHrP 1-141 to MC3T3-E1 pre-osteoblasts and LCC15-vector cells. The concentration of PTHrP was maintained for 24 hrs when added to MC3T3-E1 pre-osteoblasts, indicating that the peptide was stable for all treatment durations used in this study. In addition, in the LCC15-vector cell medium, the concentration of PTHrP was stable for 7 days. This indicated that the LCC15-MB parent cell line, which secretes negligible PTHrP, did not release soluble factors or have cell-surface enzymes that are capable of proteolysis of full-length PTHrP 1-141.

While the majority of clinical studies using PTH as a skeletal anabolic agent have used daily dosing, once-weekly administration has proven to be advantageous by circumventing the limitations of daily use while still achieving anabolic effects in bone [11]. In our study, the discovery that PTHrP treatment once every 6 days increases in vitro bone formation has several advantages: 1) it models once-weekly dosing and 2) it was achieved with lower concentrations, at which PTH 1-34 and PTHrP 107-139 have previously been shown to stimulate osteoblast differentiation and survival [12,31,32]. Since the anabolic effects of both PTH and PTHrP depend on complete clearance of the peptide between dosing, we hypothesize that due to the stability of PTHrP 1-141 in vitro, daily treatment in vitro would mimic continuous exposure to the peptide and inhibit bone formation [33]. Therefore, we investigated a less frequent dosing schedule to stimulate in vitro bone formation.

In our study, both PTHrP 1-141 and 1-86 increased in vitro bone formation after 24-hr treatments once every 6 days, supporting that once-weekly administration can achieve anabolic effects in bone [11]. The fact that PTHrP 1-141 increased in vitro bone formation in a manner equivalent to or exceeding PTHrP 1-86, combined with our experiments assessing region-specific blockade, supports that the peptide’s anabolic effects are not restricted to its N-terminus. Our results demonstrate the utility and potential advantage of local delivery of full-length PTHrP 1-141 as a skeletal anabolic agent, warranting its addition as a skeletal therapeutic.

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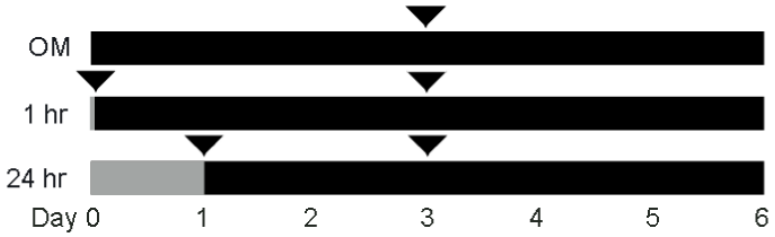

Fig. 1.

Treatment protocol for MC3T3-E1 pre-osteoblast mineralization assays with OM or 1- and 24-hr of treatment with vector, PTHrP 1-86, or PTHrP 1-141. Schematic represents (1) 6-day treatment cycle. Grey areas represent exposure to vector, PTHrP 1-86, or PTHrP 1-141 and black areas represent exposure to OM. Black arrows represent when either OM, vector, PTHrP 1-86, or PTHrP 1-141 was aspirated and cells treated with OM. Numbers indicate the day during each 6-day cycle.

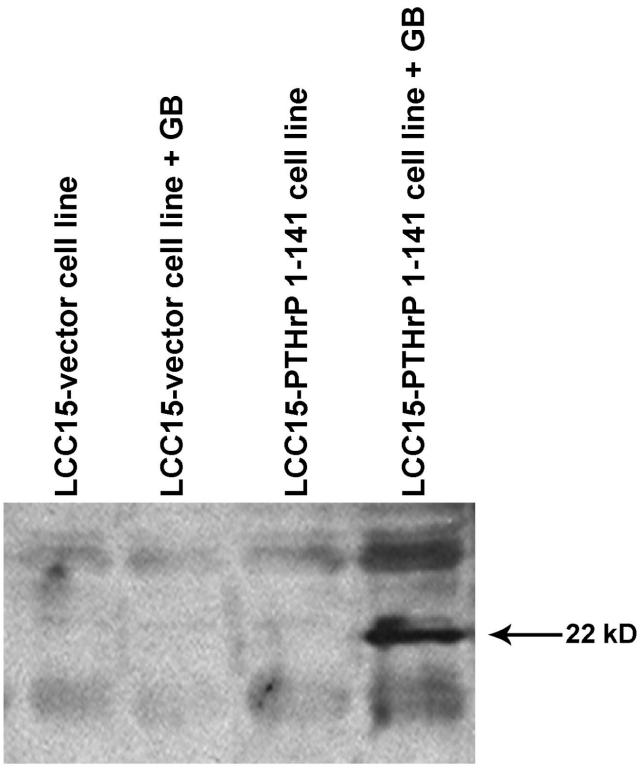

Fig. 2.
Western blot of PTHrP 1-141 in whole-cell extracts produced by only the LCC15-PTHrP 1-141 cell line (22 kDa band, far right column). From left to right, LCC15-vector and LCC15-PTHrP 1-141 cell lines and each cell line after the addition of a Golgi blocker (GB, Golgi Plug™, BD Biosciences).

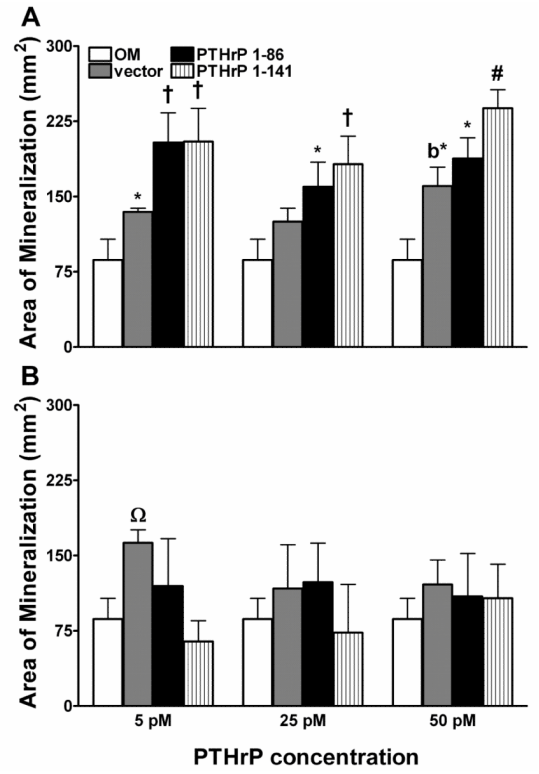

Fig. 3.

Area of mineralization at day 36 after treatment with OM, vector, PTHrP 1-86, and PTHrP 1-141 for **(A)** 24-hrs or **(B)** 1-hr once every 6 days, where PTHrP 1-141 and 1-86 significantly increased mineralization after 24-hr treatments, with minimal effect using 1-hr. In addition, PTHrP 1-141 was more potent than 1-86. Bars represent mean ± 1 standard deviation of three individual wells. Along the x-axis, pM represents the concentration of PTHrP 1-141, PTHrP 1-86, and equivalent pM vector. Within each concentration, "*" indicate a statistically significant difference from OM, "†" from OM and vector, "#" from OM, vector, and PTHrP 1-86, and "Ω" from OM and PTHrP 1-141. Between concentrations, "a" indicates a statistically significant difference from 5 pM, "b" from 25 pM, and "c" from both 5 and 25 pM.

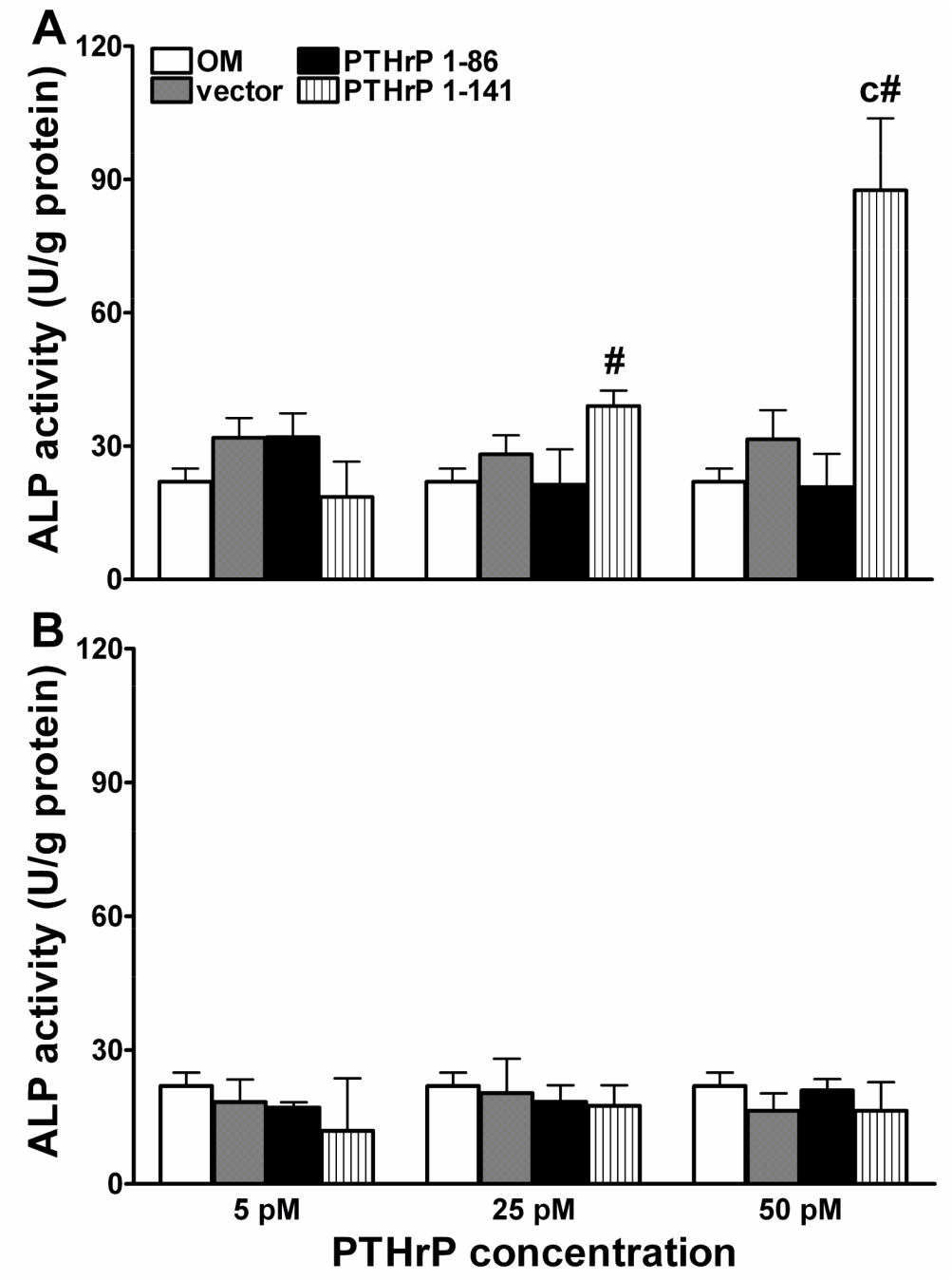

Fig. 4.

ALP activity (U/g secreted protein) at day 18 after treatment with OM, vector, PTHrP 1-86, and PTHrP 1-141 for **(A)** 24-hrs or **(B)** 1-hr once every 6 days, where treatment with PTHrP 1-141 for 24-hr, but not 1-86, resulted in a concentration-dependent increase in ALP activity, which exceeded 1-86. No effect was observed after 1-hr. See Fig. 3 for legend.

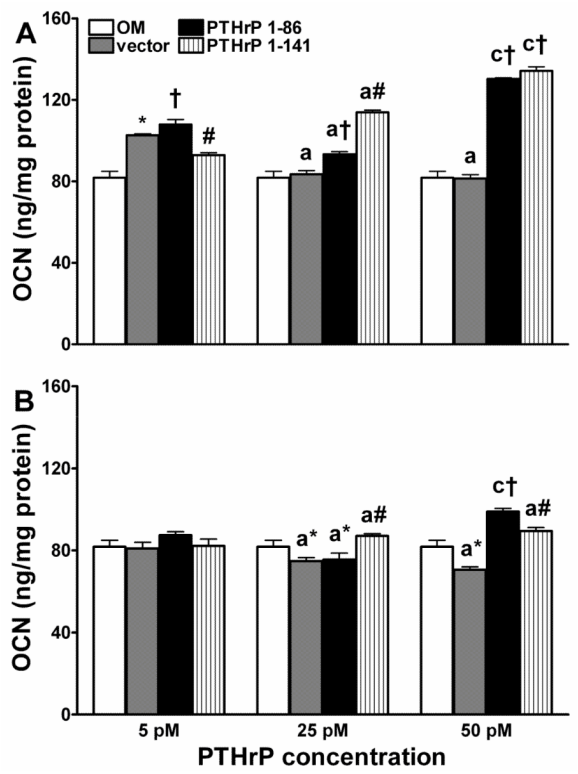

Fig. 5.

OCN concentrations (ng/mg secreted protein) at day 36 after treatment with OM, vector, PTHrP 1-86, and PTHrP 1-141 for **(A)** 24-hrs or **(B)** 1-hr once every 6 days, where treatment with both peptides for 1- or 24-hrs induced a concentration-dependent increase in OCN secretion; however, 24-hr consistently exceeded 1-hr. See Fig. 3 for legend.

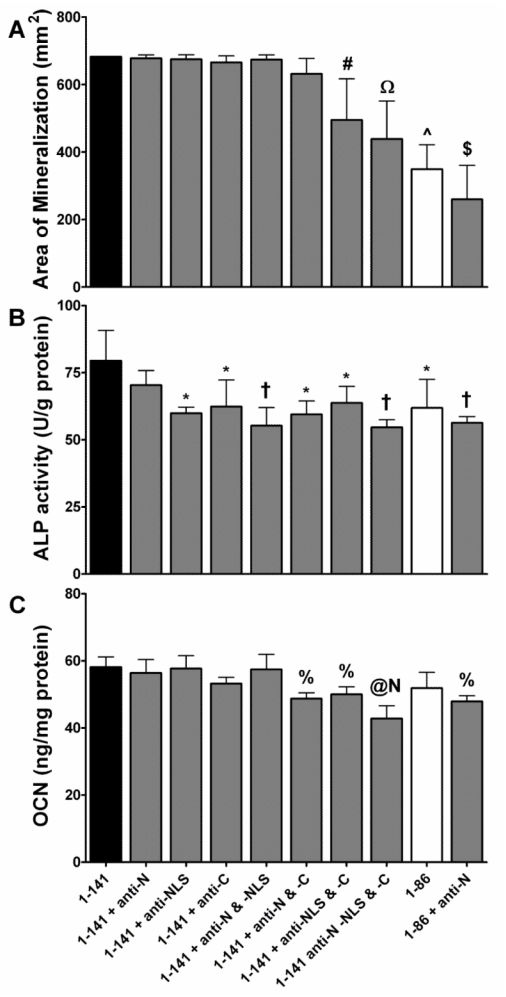

Fig. 6.

Area of mineralization **(A)**, ALP activity **(B)**, and OCN concentrations **(C)** after treatment with 50 pM of PTHrP 1-86 or 1-141 for 24-hr once every 6 days, both alone and after treating PTHrP 1-141 with each of the following individually or all possible combinations thereof: anti-N-terminal antibody, anti-NLS antibody, and anti-C-terminal antiserum. PTHrP 1-86 was treated with only anti-N-terminal antibody. Region-specific blockade reveals that the NLS and C-terminus are capable of eliciting anabolic effects distinct from, or complimentary to, the N-terminus of the peptide. Bars represent mean ± 1 standard deviation of four to six individual wells. Between treatment groups, statistically significant differences from the group in parenthesis are noted by: 1) “*” (PTHrP 1-141), 2) “†” (PTHrP 1-141 and PTHrP 1-141 + N-terminal blockade), 3) “#” (PTHrP 1-141 and all blocking combinations except N- and C-terminal blockade and complete blockade), 4) “Ω” (PTHrP 1-141 and all blocking combinations except NLS and C-terminal blockade), 5) “^” (PTHrP 1-141 and all blocking combinations except NLS and C-terminal blockade and complete blockade, 6) “@” (PTHrP 1-141 and all blocking combinations except complete blockade), and 7) “$” (PTHrP 1-141 and all blocking combinations). Specifically for **(C)**, 1) “%” (PTHrP 1-141 and PTHrP 1-141 + N-terminal blockade, PTHrP 141 + NLS blockade, and PTHrP 1-141 + N-terminus and NLS blockade and 2) “N” (PTHrP 1-86).